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(54) Title: GENE CODING FOR ANDROCTONINE, VECTOR CONTAINING SAME AND TRANSFORMED DISEASE-RESISTANT PLANTS OBTAINED

(54) Titre: GENE CODANT POUR L'ANDROCTONINE, VECTEUR LE CONTENANT ET PLANTES TRANSFORMEES OBTENUES RESISTANTES AUX MALADIES

(57) Abstract

The invention concerns a DNA sequence coding for androctonine, a vector containing same for transforming a host organism and the transformation method. More particularly the invention concerns the transformation of plant cells and plants, the drosomycine produced by the transformed plants providing them with resistance to diseases, in particular those of fungal origin.

(57) Abrégé

La présente invention a pour objet une séquence d'ADN codant pour l'androctonine, un vecteur la contenant pour la transformation d'un organisme hôte et le procédé de transformation. L'invention concerne plus particulièrement la transformation des cellules végétales et des plantes, la drosomycine produite par les plantes transformées leur conférant une résistance aux maladies, en particulier d'origine fongique.

# Gene coding for androctonine, vector containing it and disease-resistant transformed plants obtained

The present invention relates to a DNA sequence coding for androctonine, to a vector containing it for the transformation of a host organism and to the process for transforming the said organism.

The invention relates more particularly to the transformation of plant cells and plants and to the androctonine produced by the transformed plants, giving them resistance to diseases, in particular diseases of fungal origin.

There is today an increasing need to make plants resistant to diseases, in particular fungal diseases, in order to reduce, or even avoid altogether, the need for treatments with antifungal protection products, in order to protect the environment. One means of increasing this disease-resistance consists in transforming the plants so that they produce substances capable of defending them against these diseases.

Various substances of natural origin are known, in particular peptides, which have bactericidal or fungicidal properties, especially against the fungi responsible for plant diseases. However, the problem consists in finding such substances which not only can be produced by transformed plants, but also can conserve their bactericidal or fungicidal properties and confer these properties to the said plants. For the

purposes of the present invention, the terms bactericidal and fungicidal are understood to refer both to the actual bactericidal or fungicidal properties and to the bacteriostat or fungistat properties.

Androctonines are peptides produced by scorpions, in particular from the species *Androctonus australis*. An androctonine and its preparation by chemical synthesis are described by Ehret-Sabatier et al., along with its *in vitro* antifungal and antibacterial properties.

The androctonine genes have now been identified, and it has also been found that they can be inserted into a host organism, in particular a plant, in order to express an androctonine, both for the preparation and isolation of this androctonine and to give the said host organism properties of resistance to fungal diseases and to diseases of bacterial origin, thereby providing a particularly advantageous solution to the problem outlined above.

The subject of the invention is thus, firstly, a nucleic acid fragment coding for an androctonine, a chimeric gene comprising the said fragment coding for an androctonine and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular in plants, and a vector for transforming host organisms containing this chimeric gene, and the host organism transformed.

The invention also relates to a transformed plant cell containing at least one nucleic acid fragment coding for an androctonine, and to a disease-resistant plant containing the said cell, in particular a plant regenerated from this cell. Lastly, the invention relates to a process for cultivating transformed plants according to the invention.

According to the invention, the term androctonine is understood to refer to any peptide which can be produced by and isolated from scorpions, in particular from the species *Androctonus australis*, these peptides comprising at least 20 amino acids, preferably at least 25, and 4 cysteine residues which form disulphide bridges between themselves.

Advantageously, the androctonine essentially comprises the peptide sequence of general formula (I) below:

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae

(I)

in which

Xaa represents a peptide residue comprising at least 1 amino acid,

Xab represents a peptide residue of 5 amino acids,

Xac represents a peptide residue of 5 amino acids,

Xad represents a peptide residue of 3 amino acids, and

Xae represents a peptide residue comprising at least 1 amino acid.

Advantageously, Xab and/or Xad and/or Xae comprise at least one basic amino acid, preferably 1.

According to the invention, the term basic amino acids is understood to refer to amino acids chosen from lysine, asparagine and homoasparagine.

Preferably,

Xaa represents the peptide sequence Xaa'-Val, in which Xaa' represents $NH_2$ or a peptide residue comprising at least 1 amino acid, and/or

Xab represents the peptide sequence -Arg-Xab'-Ile, in which Xab' represents a peptide residue of 3 amino acids, and/or

Xac represents the peptide sequence -Arg-Xac'-Gly-, in which Xac' represents a peptide residue of 3 amino acids, and/or

Xad represents the peptide sequence -Tyr-Xad'-Lys, in which Xad' represents a peptide residue of 1 amino acid, and/or

Xae represents the peptide sequence -Thr-Xae', in which Xae' represents COOH or a peptide residue comprising at least 1 amino acid.

Preferably,

Xaa' represents the peptide sequence Arg-Ser-, and/or

Xab' represents the peptide sequence -Gln-Ile-Lys-, and/or

Xac' represents the peptide sequence -Arg-Arg-Gly-, and/or

Xad' represents the peptide residue -Tyr-, and/or

Xae' represents the peptide sequence -Asn-Arg-Pro-Tyr.

According to a preferred embodiment of the

invention, androctonine is represented by the peptide sequence of 25 amino acids described by the sequence identifier No. 1 (SEQ ID NO. 1) and the homologous peptide sequences.

The term homologous peptide sequences is understood to refer to any equivalent sequence comprising at least 65% homology with the sequence represented by the sequence identifier No. 1, it being understood that the 4 cysteine residues and the number of amino acids separating them remain identical, certain amino acids being replaced with different but equivalent amino acids on sites which do not induce a substantial change in the antifungal or antibacterial activity of the said homologous sequence. Preferably, the homologous sequences comprise at least 75% homology, more preferably at least 85% homology and even more preferably 90% homology.

The $NH_2$-terminal residue of androctonine can exhibit a post-translational modification, for example an acetylation, while the C-terminal residue can exhibit a post-translational modification, for example an amidation.

The expression peptide sequence essentially comprising the peptide sequence of general formula (I) is understood to refer not only to the sequences defined above, but also to such sequences comprising, at one or other of their ends or at both ends, peptide residues required for their expression and targeting in

a host organism, in particular a plant cell or plant.

This in particular concerns a "peptide-androctonine" or "androctonine-peptide", advantageously "peptide-androctonine", fusion peptide whose cleavage by the enzymatic systems of the plant cells allows the release of the androctonine defined above. The peptide fused to androctonine can be a signal peptide or a transit peptide which allows the production of androctonine to be controlled and oriented specifically in one part of the host organism, in particular of the plant cell or plant, such as, for example, the cytoplasm or the cell membrane, or in the case of plants, in a specific type of cell or tissue compartment or in the extracelluar matrix.

According to one embodiment, the transit peptide can be a chloroplast-addressing signal or a mitochondrion-addressing signal, which is then cleaved off in the chloroplasts or the mitochondria.

According to another embodiment of the invention, the signal peptide can be an N-terminal signal or "prepeptide", optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a vacuole-addressing peptide or "propeptide". The endoplasmic reticulum is the site at which maturation operations on the protein produced, such as, for example, cleavage of the signal peptide, are undertaken by the "cell machinery".

The transit peptides can be single or double,

and, in this case, optionally separated by an intermediate sequence, i.e. one comprising, in the direction of transcription, a sequence coding for a transit peptide of a plant gene which codes for a plastid localization enzyme, a portion of sequence of the N-terminal mature portion of a plant gene coding for a plastid localization enzyme, and then a sequence coding for a second transit peptide of a plant gene coding for a plastid localization enzyme, as described in patent application EP 0,508,909.

As transit peptide which is useful according to the invention, mention may be made in particular of the signal peptide of the tobacco PR-1α gene (WO 95/19443), represented with its coding sequence by the sequence identifier No. 2 (SEQ ID NO. 2) and fused to androctonine by the sequence identifier No. 3 (SEQ ID NO. 3), in particular corresponding to the fusion protein corresponding to bases 12 to 176 of this sequence, in particular when the androctonine is produced by plant cells or plants, or the precursor of Mat α1 factor when the androctonine is produced in yeasts.

The present invention thus relates, firstly, to a nucleic acid fragment, in particular a DNA fragment, coding for the androctonine defined above. According to the invention, this can be a fragment isolated from *Androctonus australis*, or alternatively a derived fragment, adapted for the expression of

androctonine in the host organism in which the peptide will be expressed. The nucleic acid fragment can be obtained according to the standard methods for isolation and purification, or alternatively by synthesis according to the usual techniques of successive hybridizations of synthetic oligonucleotides. These techniques are described in particular by Ausubel et al.

According to the present invention, the expression "nucleic acid fragment" is understood to refer to a nucleotide sequence which can be of DNA or RNA type, preferably of DNA type, in particular cDNA, especially of double-stranded type.

According to one embodiment of the invention, the nucleic acid fragment coding for androctonine is the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO. 1), a homologous sequence or a sequence complementary to the said sequence, more particularly the coding portion of this SEQ ID NO. 1, corresponding to bases 1 to 75.

According to the invention, the term "homologous" is understood to refer to a nucleic acid fragment having one or more sequence modifications when compared with the nucleotide sequence described by the sequence identifier No. 1 coding for androctonine. These modifications can be obtained according to the usual mutation techniques, or alternatively by selecting the synthetic oligonucleotides used in the

preparation of the said sequence by hybridization. With regard to multiple combinations of nucleic acids which can lead to the expression of the same amino acid, the differences between the reference sequence described by the sequence identifier No. 1 and the homologous sequence can be considerable, and all the more so when it concerns a DNA fragment less than 100 nucleic acids in size, which can be produced by synthesis. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80% and more preferably at least 90%. These modifications are generally neutral, i.e. they do not affect the primary sequence of the resulting androctonine.

The present invention also relates to a chimeric gene (or expression cassette) comprising a coding sequence and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular plant cells or plants, these elements being functionally linked to the said coding sequence, the said coding sequence comprising at least one DNA fragment coding for androctonine as defined above (including the "peptide-androctonine" or "androctonine-peptide" fusion peptide).

The term host organism is understood to refer to any lower-order or higher-order monocellular or multicellular organism into which the chimeric gene according to the invention can be introduced, for the

production of androctonine. Such organisms are, in particular, bacteria, for example *E. coli*, yeasts, in particular yeasts of the genera *Saccharomyces* or *Kluyveromyces, Pichia*, fungi, in particular *Aspergillus*, a baculovirus, or, preferably, plant cells and plants.

According to the invention, the term "plant cell" is understood to refer to any plant-derived cell which can constitute undifferentiated tissues such as calli, differentiated tissues such as embryos, plant portions, plants or seeds.

According to the invention, the term "plant" is understood to refer to any differentiated multicellular organism capable of photosynthesis, in particular monocotyledons or dicotyledons, more particularly crop plants which may or may not be intended for human or animal consumption, such as corn, wheat, rapeseed, soybean, rice, sugar cane, beetroot, tobacco, cotton, etc.

The regulation elements required for the expression of the DNA fragment coding for androctonine are well known to those skilled in the art as a function of the host organism. They comprise in particular promoter sequences, transcription activators, transit peptides and termination sequences, including start and stop codons. The means and methods for identifying and selecting the regulation elements are well known to those skilled in the art.

For the transformation of microorganisms such as yeasts or bacteria, the regulation elements are well known to those skilled in the art and comprise, in particular, promoter sequences, transcription activators, transit peptides, termination sequences and start and stop codons.

In order to direct the expression and secretion of the peptide in the yeast culture medium, a DNA fragment coding for heliomycin is incorporated into a shuttle vector which comprises the following elements:

- markers which allow the transformants to be selected,
- a nucleic acid sequence which allows replication (origin of replication) of the plasmid in the yeast,
- a nucleic acid sequence which allows replication (origin of replication) of the plasmid in *E. coli*,
- an expression cassette consisting of

(1) a promoter regulation sequence,

(2) a sequence coding for a signal peptide (or prepeptide) combined with an addressing peptide (or propeptide),

(3) a polyadenylation or terminator regulation sequence.

These elements have been described in several publications, including Reichhart et al., 1992, Invert. Reprod. Dev., 21, pp. 15-24 and Michaut et al., 1996, FEBS Letters, 395, pp. 6-10.

Preferably, yeasts from the species *S.*

*cerevisiae* are transformed with the expression plasmid by the lithium acetate method (Ito et al., 1993, J. Bacteriol, 153, pp. 163-168).

The invention relates more particularly to the transformation of plants. As promoter regulation sequence in plants, it is possible to use any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for the small subunit of ribulose biscarboxylase/oxygenase (RuBisCO) or of a plant virus gene such as, for example, that of cauliflower mosaic virus (CAMV 19S or 35S), or a promoter which can be induced by pathogens such as tobacco PR-1a, it being possible to use any suitable known promoter. Preferably, use is made of a promoter regulation sequence which favours the overexpression of the coding sequence in a constitutive manner or induced by the attack of a pathogen, such as, for example, that comprising at least one histone promoter as described in patent application EP 0,507,698.

According to the invention, it is also possible to use, in combination with the promoter regulation sequence, other regulation sequences which are located between the promoter and the coding sequence, such as transcription activators ("enhancers"), such as, for example, the tobacco mosaic virus (TMV) translation activator described in patent

application WO 87/07644, or the tobacco etch virus (TEV) translation activator described by Carrington & Freed.

As polyadenylation or terminator regulation sequence, it is possible to use any corresponding sequence of bacterial origin, such as, for example, the nos terminator of *Agrobacterium tumefaciens*, or alternatively of plant origin, such as, for example, a histone terminator as described in patent application EP 0,633,317.

According to the present invention, the chimeric gene can also be combined with a selection marker adapted to the transformed host organism. Such selection markers are well known to those skilled in the art. Such a marker may be an antibiotic-resistance gene or alternatively a herbicide-tolerance gene for plants.

The present invention also relates to a cloning or expression vector for the transformation of a host organism containing at least one chimeric gene as defined above. Besides the above chimeric gene, this vector comprises at least one origin of replication and, where appropriate, a suitable selection marker. This vector can consist of a plasmid, a cosmid, a bacteriophage or a virus, which are transformed by introducing the chimeric gene according to the invention. Depending on the host organism to be transformed, such transformation vectors are well known

to those skilled in the art and are widely described in the literature.

For the transformation of plant cells or plants, such a vector is, in particular, a virus which can be used for the transformation of the plants developed and also containing its own replication and expression elements. Preferably, the vector for transforming the plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a process for transforming host organisms, in particular plant cells, by incorporating at least one nucleic acid fragment or one chimeric gene as defined above, it being possible for this transformation to be obtained by any suitable known means, which is amply described in the specialized literature, and in particular the references cited in the present application, more particularly by means of the vector according to the invention.

One series of methods consists in bombarding cells, protoplasts or tissues with particles to which the DNA sequences are attached. Another series of methods consists in using, as a means of transfer into the plant, a chimeric gene inserted into a Ti plasmid of *Agrobacterium tumefaciens* or an Ri plasmid of *Agrobacterium rhizogenes*.

Other methods can be used, such as microinjection or electroporation, or alternatively

direct precipitation using PEG.

A person skilled in the art will select the appropriate method as a function of the nature of the host organism, in particular the plant cell or plant.

The subject of the present invention is also transformed host organisms, in particular plant cells or plants, containing an effective amount of a chimeric gene comprising a sequence coding for the androctonine defined above.

The subject of the present invention is also plants containing transformed cells, in particular plants regenerated from the transformed cells. The regeneration is obtained by any suitable process which depends on the nature of the species, as described, for example, in the above references.

For the processes for transforming plant cells and for regenerating plants, mention will be made in particular of the following patents and patent applications: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

The subject of the present invention is also

the transformed plants obtained from the cultivating and/or crossing of the above regenerated plants, as well as the seeds of transformed plants.

The plants thus transformed are resistant to certain diseases, in particular to certain fungal or bacterial diseases. Consequently, the DNA sequence coding for androctonine can be inserted with the main aim of producing plants that are resistant to the said diseases, since androctonine is effective against fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

The chimeric gene may also advantageously be combined with at least one selection marker, such as one or more herbicide-tolerance genes.

The DNA sequence coding for androctonine can also be inserted as a selection marker during the transformation of plants with other sequences coding for other peptides or proteins of interest, such as, for example, herbicide-tolerance genes.

Such herbicide-tolerance genes are well known to those skilled in the art and are described in particular in patent applications EP 115,673, WO 87/04181, EP 337,899, WO 96/38567 or WO 97/04103.

Needless to say, the transformed cells and

plants according to the invention can also comprise the sequence coding for androctonine, other heterologous sequences coding for proteins of interest, such as other complementary peptides capable of giving the plant resistance to other diseases of bacterial or fungal origin, and/or other sequences coding for herbicide-tolerance proteins, in particular defined above and/or other sequences coding for insect-resistance proteins, such as the *Bt* proteins in particular.

The other sequences can be inserted using the same vector comprising the chimeric gene according to the invention, which comprises a sequence coding for androctonine, and comprising at least one other gene comprising another sequence coding for another peptide or protein of interest.

They can also be inserted using another vector comprising at least the said other sequence, according to the usual techniques defined above.

The plants according to the invention can also be obtained by crossing parents, one carrying the gene according to the invention coding for androctonine, the other carrying a gene coding for at least one other peptide or protein of interest.

Among the sequences coding for other antifungal peptides, mention may be made of the one coding for drosomycin, described in patent application Fr 2,725,992 and by Fehlbaum et al., (1994), and in the

unpublished patent application FR 97/09115 filed on 24 July 1997.

Lastly, the present invention relates to a process for cultivating transformed plants according to the invention, the process consisting in planting the seeds of the said transformed plants in an area of a cultivation environment, in particular a field, which is suitable for cultivating the said plants, in applying an agrochemical composition to the said area, without substantially affecting the said transformed seeds or plants, and then in harvesting the plants cultivated when they reach the desired maturity, and optionally in separating the seeds from the harvested plants.

According to the invention, the term agrochemical composition is understood to refer to any agrochemical composition comprising at least one active product having either herbicidal, fungicidal, bactericidal, virucidal or insecticidal activity.

According to a preferred embodiment of the cultivation process according to the invention, the agrochemical composition comprises at least one active product having at least a fungicidal and/or bactericidal activity, more preferably having an activity complementary to that of the androctonine produced by the transformed plants according to the invention.

According to the invention, the expression

product having activity complementary to that of androctonine is understood to refer to a product having a complementary spectrum of activity, i.e. a product which will be active against attacks by androctonine-insensitive contaminants (fungi, bacteria or viruses), or alternatively a product whose spectrum of activity totally or partially covers that of androctonine, and whose dose of application will be substantially reduced on account of the presence of the androctonine produced by the transformed plant.

Lastly, cultivation of the transformed host organisms allows the large-scale production of androctonine. The subject of the present invention is thus also a process for preparing androctonine, comprising the steps of cultivating the transformed host organism comprising a gene coding for androctonine as defined above in an appropriate cultivation environment, followed by the extraction and total or partial purification of the androctonine obtained.

The examples below make it possible to illustrate the invention, the preparation of the sequence coding for androctonine, the chimeric gene, the integration vector and the transformed plants. The attached Figures 1 to 5 describe schematic structures of certain plasmids prepared for the construction of the chimeric genes. In these figures, the various restriction sites are marked in *italics*.

**Example 1: Construction of the chimeric genes**

All the techniques used below are standard laboratory techniques. The detailed procedures for these techniques are described in particular in Ausubel et al.

**pRPA-MD-P: Creation of a plasmid containing the signal peptide for the tobacco PR-1a gene.**

The two complementary synthetic oligonucleotides Oligo 1 and Oligo 2 below are hybridized at 65°C for 5 minutes and then by slowly decreasing the temperature to 30°C over 30 min.

Oligo 1: 5' GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC
ATCTTTCCTT CTTGTGTCTA CTCTTCTTCT TTTCC 3'

Oligo 2: 5' TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA
GAAGAGTAGA CACAAGAAGG AAAGATGGAA GC 3'

After hybridization between Oligo 1 and Oligo 2, the remaining single-stranded DNA serves as a matrix for the klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes *SacII* and *NaeI* and cloned in the plasmid pBS II SK(-) (Stratagene) digested with the

same restriction enzymes. A clone comprising the region coding for the signal peptide of the tobacco PR-1a gene (SEQ ID NO. 2) is thus obtained.

**pRPA-PS-PR1a-andro: Creation of a sequence coding for androctonine fused to the PR-1a signal peptide without an untranscribed 3' region.**

The two complementary synthetic oligonucleotide sequences Oligo 3 and Oligo 4 are hybridized according to the operating conditions described for pRPA-MD-P.

Oligo 3: 5' AGGTCCGTGT GCAGGCAGAT CAAGATCTGC AGGAGGAGGG GTGG 3'

Oligo 4: 5' CCGGATCCGT CGACACGTTC GCCTCGCCGA GCTCAGTATG GCCTGTTAGT GCACTTGTAG TAGCAACCAC CCCTCCTCCT GCAGATCTTG ATCTGCC 3'

After hybridization between Oligo 3 and Oligo 4, the remaining single-stranded DNA serves as a matrix for the klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. This double-stranded oligonucleotide containing the portion coding for androctonine (SEQ ID NO. 1) is then cloned directly in the plasmid pRPA-MD-P, which was digested with the

restriction enzyme *NaeI*. The correct orientation of the clone obtained is verified by sequencing. A clone comprising the region coding for the PR-1a-androctonine fusion protein, located between the *NcoI* restriction site at the N-terminal end and the *ScaI*, *SacII* and *BamHI* restriction sites at the C-terminal end (SEQ ID NO. 3), is thus obtained.

**pRPA-RD-238: Creation of an expression vector in plants comprising the sequence coding for the PR-1a androctonine fusion protein.**

The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr. Jim Carrington (Texas A&M University, not described). This plasmid, whose schematic structure is represented in Figure 1, contains the duplicated CaMV 35S promoter isolated from cauliflower mosaic virus (CaMV 2x35S promoter; Odell et al., 1985) which directs the expression of an RNA containing a 5' untranslated sequence of tobacco etch virus (TEV 5' UTR; Carrington and Freed, 1990), the *E. coli* β-glucuronidase gene (GUS; Jefferson et al., 1987) followed by the CaMV RNA 35S polyadenylation site (CaMV polyA; Odell et al., 1985).

The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI* and the main DNA fragment is purified. The plasmid pRPA-PS-PR1a-andro is digested with the restriction enzymes *NcoI* and *BamHI* and the small DNA fragment containing the region coding

for the PR-1a-androctonine fusion protein is purified. The two purified DNA fragments are then linked together in an expression cassette in the plants which synthesizes a PR-1a-androctonine fusion protein. The schematic structure of this expression cassette is represented in Figure 2. "PR-1a-androctonine" represents the region coding for the PR-1a-androctonine fusion protein of pRPA-RD-230. The androctonine is transported to the plant's extracelluar matrix by the action of the PR-1a peptide signal.

**pRPA-RD-195: Creation of a plasmid containing a modified multiple cloning site.**

The plasmid pRPA-RD-195 is a plasmid derived from pUC-19 which contains a modified multiple cloning site. The complementary synthetic oligonucleotides Oligo 5 and Oligo 6 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 5: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG CATGC 3'

Oligo 6: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC TAGAGG 3'

The double-stranded oligonucleotide obtained is then inserted into pUC-19, which was predigested

with the restriction enzymes *EcoRI* and *HindIII* and made blunt at the ends using the klenow fragment of *E. coli* DNA polymerase 1. A vector containing multiple cloning sites to facilitate the introduction of the expression cassettes into an *Agrobacterium tumefaciens* vector plasmid is obtained. The schematic structure of this multiple cloning site is represented in Figure 3.

**pRPA-RD-233: Introduction of the PR-1a-androctonine expression cassette from pRPA-RD-230 into pRPA-RD-195.**

The plasmid pRPA-RD-230 is digested with the restriction enzyme *HindIII*. The DNA fragment containing the PR-1a-androctonine expression cassette is purified. The purified fragment is then inserted into pRPA-RP-195, which was predigested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase.

**pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the bromoxynil-tolerance gene from pRPA-BL-237 (EP 0,508,909).**

The bromoxynil-tolerance gene is isolated from pRPA-BL-237 by means of a PCR gene amplification. The fragment obtained has blunt ends, and is cloned in the pRPA-BL-150A *EcoRI* site, the ends of which were made blunt by the action of klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector which contains the bromoxynil-tolerance gene

close to its right-hand end, a kanamycin-tolerance gene close to its left-hand end and a multiple cloning site between these two genes is obtained.

The schematic structure of pRPA-RD-174 is represented in Figure 4. In this figure, "nos" represents the polyadenylation site of *Agrobacterium tumefaciens* nopaline synthase (Bevan et al., 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan et al., 1983), "NPT II" represents the neomycin phosphotransferase gene of the Tn5 transposon of *E. coli* (Rothstein et al., 1981), "35S pro" represents the 35S promoter isolated from cauliflower mosaic virus (Odell et al., 1985), "BRX" represents the nitrilase gene isolated from *K. ozaenae* (Stalker et al., 1988), "RB" and "LB" represent, respectively, the right-hand and left-hand ends of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

**pRPA-RD-184: Addition of a new, unique restriction site into pRPA-RD-174.**

The complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 7: 5' CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC
CCCGGCGCGC CTAGGTGTGT GCTCGAGGGC CCAACCTCAG
TACCTGGTTC AGG 3'

Oligo 8: 5' CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA
CACCTAGGCG CGCCGGGGCC GCGTTTAAAC TTAATTAAGT
GTGGCCTGAC TGG 3'

The hybridized double-stranded oligonucleotide (96 base pairs) is purified after separation on agarose gel (3% Nusieve, FMC). The plasmid pRPA-RD-174 is digested with the restriction enzyme *XmaI* and the main DNA fragment is purified. The two DNA fragments obtained are then linked together.

A plasmid derived from pRPA-RD-174 is obtained, comprising other restriction sites between the bromoxynil-tolerance gene and the selection marker kanamycin gene.

The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 5, in which the terms "nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB" and "LB" have the same meanings as in Figure 4.

**<u>pRPA-RD-236:</u> Creation of an *Agrobacterium tumefaciens* vector containing the gene construct coding for androctonine directed towards the extracellular matrix.**

The plasmid pRPA-RD-233 is digested with the restriction enzymes *PmeI* and *AscI* and the DNA fragment containing the PR-1a-androctonine gene is purified. The plasmid pRPA-RD-184 is digested with the same restriction enzymes. The DNA fragment containing the PR-1a-androctonine expression cassette is then inserted

into pRPA-RD-184. An *Agrobacterium tumefaciens* vector containing the sequence coding for the PR-1a-androctonine fusion protein is thus obtained, which leads to the expression of androctonine in the plant's extracellular matrix.

**Example 2: Tolerance to herbicides of transformed tobacco plants.**

**2.1- Transformation**

The vector pRPA-RD-236 is introduced into the *Agrobacterium tumefaciens* strain EHA101 (Hood et al., 1987) carrying the cosmid pTVK291 (Komari et al., 1986). The transformation technique is based on the procedure by Horsh et al. (1985).

**2.2- Regeneration**

Regeneration of the tobacco plant PBD6 (obtained from SEITA France) from foliar explants is carried out on Murashige-Skoog (MS) base medium comprising 30 g/l of sucrose and 200 µg/ml of kanamycin. The foliar explants are taken from plants cultivated in a greenhouse or *in vitro* and regenerated according to the foliar disc technique (Horsh et al., 1985) in three successive steps: the first step comprises induction of the shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 2 weeks. The shoots formed during this step are then grown for 10 days by

cultivating on MS medium supplemented with 30 g/l of sucrose but containing no hormone. Next, the shoots which have grown are taken and cultivated on an MS rooting medium with half the content of salts, vitamins and sugar and containing no hormone. After about 2 weeks, the rooted shoots are placed in a greenhouse.

2.3- **Tolerance to bromoxynil**

Twenty transformed plants were regenerated and placed in a greenhouse for the construction of pRPA-RD-236. These plants were treated in the greenhouse, at the 5-leaf stage, with aqueous Pardner suspension corresponding to 0.2 kg of bromoxynil active material per hectare.

All the plants showing complete tolerance to bromoxynil are then used in various experiments which show that the expression of androctonine by the transformed plants makes them resistant to fungal attack.

**REFERENCES**

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CLAIMS

1. Nucleic acid fragment, characterized in that it comprises a nucleic acid sequence coding for an androctonine.

2. Nucleic acid fragment according to claim 1, characterized in that it is a sequence of DNA.

3. Nucleic acid fragment according to either of claims 1 and 2, characterized in that the androctonine consists of a peptide which can be produced by and isolated from scorpions, in particular from the species *Androctonus australis*, the said peptide comprising at least 20 amino acids, preferably at least 25 amino acids, and 4 cysteine residues which form disulphide bridges between themselves.

4. Nucleic acid fragment according to one of claims 1 to 3, characterized in that the androctonine essentially comprises the peptide sequence of general formula (I) below

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae

(I)

in which

Xaa represents a peptide residue comprising at least 1 amino acid,

Xab represents a peptide residue of 5 amino acids,

Xac represents a peptide residue of 5 amino acids,

Xad represents a peptide residue of 3 amino acids, and

Xae represents a peptide residue comprising at least 1 amino acid.

5. Nucleic acid fragment according to claim 4, characterized in that Xab and/or Xad and/or Xae comprise at least one basic amino acid.

6. Nucleic acid fragment according to claim 5, characterized in that the basic amino acids are chosen from lysine, asparagine and homoasparagine.

7. Nucleic acid fragment according to one of claims 4 to 6, characterized in that

Xaa represents the peptide sequence Xaa'-Val, in which Xaa' represents $NH_2$ or a peptide residue comprising at least 1 amino acid, and/or

Xab represents the peptide sequence -Arg-Xab'-Ile, in which Xab' represents a peptide residue of 3 amino acids, and/or

Xac represents the peptide sequence -Arg-Xac'-Gly-, in which Xac' represents a peptide residue of 3 amino acids, and/or

Xad represents the peptide sequence -Tyr-Xad'-Lys, in which Xad' represents a peptide residue of 1 amino acid, and/or

Xae represents the peptide sequence -Thr-Xae', in which Xae' represents COOH or a peptide residue comprising at least 1 amino acid.

8. Nucleic acid fragment according to claim 7, characterized in that

Xaa' represents the peptide sequence -Arg-Ser-, and/or

Xab' represents the peptide sequence -Gln-Ile-Lys-, and/or

Xac' represents the peptide sequence -Arg-Arg-Gly-, and/or

Xad' represents the peptide residue -Tyr-, and/or

Xae' represents the peptide sequence -Asn-Arg-Pro-Tyr.

9. Nucleic acid fragment according to one of claims 1 to 8, characterized in that the androctonine is represented by the peptide sequence of 25 amino acids described by the sequence identifier No. 1 (SEQ ID NO. 1) and the homologous peptide sequences.

10. Nucleic acid fragment according to claim 9, characterized in that it is represented by the sequence identifier No. 1 (SEQ ID NO. 1), a homologous sequence or a sequence complementary to the said sequence, more particularly the coding portion of this SEQ ID NO. 1, corresponding to bases 1 to 75.

11. Nucleic acid fragment, characterized in that it comprises a nucleic acid sequence coding for a "peptide-androctonine" or "androctonine-peptide", advantageously "peptide-androctonine", fusion peptide, the androctonine being defined according to one of claims 1 to 9.

12. Nucleic acid fragment according to claim 11, characterized in that the peptide fused to androctonine is a signal peptide or a transit peptide.

13. Nucleic acid fragment according to claim 12, characterized in that the transit peptide is a chloroplast-addressing signal or a mitochondrion-addressing signal.

14. Nucleic acid fragment according to claim 12, characterized in that the signal peptide is an N-terminal signal or "prepeptide", optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a vacuole-addressing peptide or "propeptide".

15. Nucleic acid fragment according to claim 14, characterized in that the signal peptide is the signal peptide of the tobacco PR-1α gene.

16. Nucleic acid fragment according to claim 15, characterized in that the "peptide-androctonine" fusion peptide is represented by the sequence identifier No. 3 (SEQ ID NO. 3).

17. Nucleic acid fragment according to claim 16, characterized in that the coding sequence is represented by the sequence identifier No. 3 (SEQ ID NO. 3), a homologous sequence or a complementary sequence, more particularly the coding portion of this SEQ ID NO. 3, corresponding to bases 12 to 176 of this sequence.

18. "Peptide-androctonine" or "androctonine-peptide", preferably "peptide-androctonine", fusion protein, characterized in that it is defined according to claims 11 to 16.

19. Chimeric gene comprising a coding sequence and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular plant cells or plants, these

elements being functionally linked to the said coding sequence, characterized in that the said coding sequence comprises at least one DNA fragment coding for androctonine as defined according to claims 1 to 17.

20. Chimeric gene according to claim 19, characterized in that the host organism is chosen from bacteria, for example *E. coli*, yeasts, in particular yeasts of the genera *Saccharomyces* or *Kluyveromyces*, *Pichia*, fungi, in particular *Aspergillus*, a baculovirus, and plant cells and plants.

21. Chimeric gene according to either of claims 19 and 20, characterized in that it is combined with a selection marker adapted to the transformed host organism.

22. Cloning or expression vector for the transformation of a host organism, characterized in that it comprises at least one chimeric gene as defined according to claims 19 to 21.

23. Process for transforming host organisms, in particular plant cells, by incorporating at least one nucleic acid fragment or one chimeric gene as defined in claims 19 to 21.

24. Process according to claim 23, characterized in that the chimeric gene is incorporated by means of the vector according to claim 22.

25. Process according to either of claims 23 and 24, characterized in that the host organism is chosen from bacteria, for example *E. coli*, yeasts, in

particular yeasts of the genera *Saccharomyces* or *Kluyveromyces, Pichia*, fungi, in particular *Aspergillus*, a baculovirus, and plant cells and plants.

26. Process according to claim 25, characterized in that the host organism is a plant cell.

27. Process according to claim 26, characterized in that plants are regenerated from transformed plant cells.

28. Transformed host organism, in particular plant cell or plant, characterized in that it comprises a chimeric gene defined according to one of claims 19 to 21.

29. Host organism according to claim 28, characterized in that it is chosen from bacteria, for example *E. coli*, yeasts, in particular yeasts of the genera *Saccharomyces* or *Kluyveromyces, Pichia*, fungi, in particular *Aspergillus*, a baculovirus, and plant cells and plants.

30. Plants, characterized in that they comprise transformed plant cells according to claim 29.

31. Plant according to claim 30, characterized in that it is regenerated from transformed plant cells.

32. Plant, characterized in that it is obtained from the cultivating and/or crossing of the regenerated plants according to claim 31.

33. Plant according to one of claims 30 to

32, characterized in that it is chosen from corn, wheat, rapeseed, soybean, rice, sugar cane, beetroot, tobacco and cotton.

34. Plant according to one of claims 30 to 33, characterized in that it is resistant to fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola*, *Cladosporium*, in particular *Cladosporium herbarum*, *Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi*.

35. Plant seeds according to one of claims 30 to 34.

36. Process for cultivating transformed plants according to one of claims 30 to 34, or obtained by the process according to claim 27, the said process consisting in planting the seeds of the said transformed plants in an area of a cultivation environment, in particular a field, which is suitable for cultivating the said plants, in applying an agrochemical composition to the said area, without substantially affecting the said transformed seeds or plants, and then in harvesting the plants cultivated when they reach the desired maturity, and optionally in separating the seeds from the harvested plants.

37. Process according to claim 36, characterized in that the agrochemical composition comprises at least one active product having at least a

fungicidal and/or bactericidal activity.

38. Process according to claim 37, characterized in that the active product has an activity complementary to that of the androctonine produced by the transformed plants.

39. Process for preparing the androctonine defined according to one of claims 1 to 18, comprising the steps of cultivating the transformed host organism defined according to either of claims 28 and 29 in an appropriate cultivation environment, followed by the extraction and total or partial purification of the androctonine obtained.

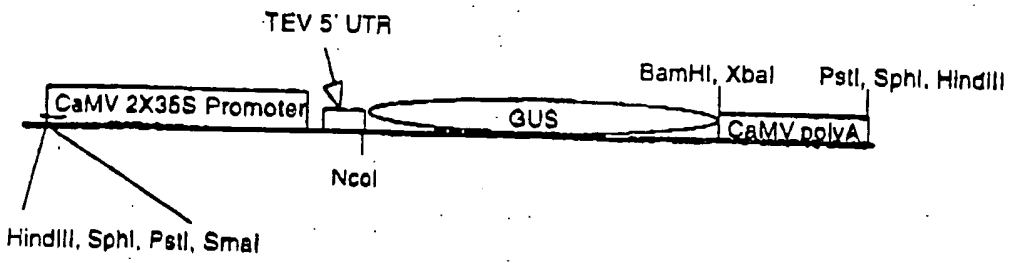


Fig. 1

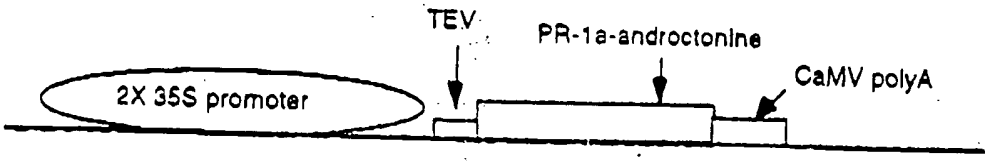


Fig. 2

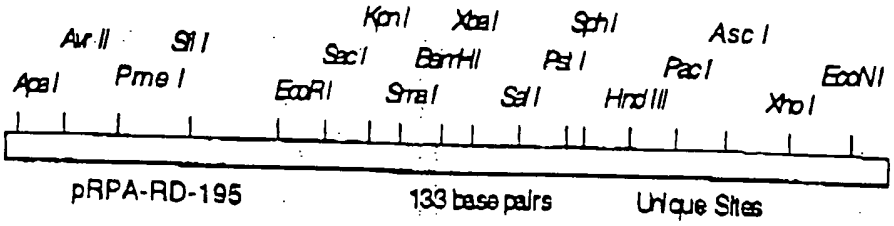


Fig. 3

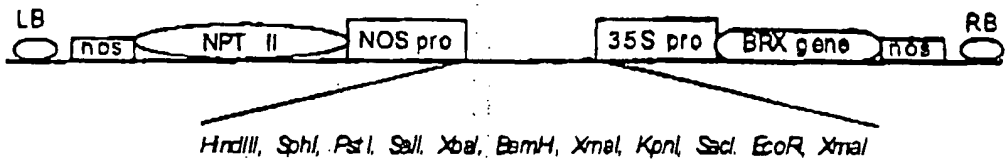
LB
nos
NPT II
NOS pro
35S pro
BRX gene
nos
RB
HindIII, SphI, PstI, SalI, XbaI, BamH, XmaI, KpnI, SacI, EcoR, XmaI

Fig. 4

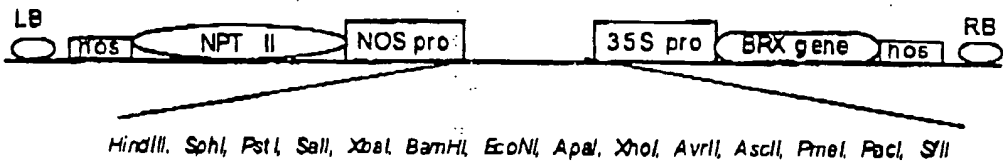
LB
nos
NPT II
NOS pro
35S pro
BRX gene
nos
RB
HindIII, SphI, PstI, SalI, XbaI, BamHI, EcoNI, ApaI, XhoI, AvrII, AscII, PmeI, PacI, SfiI

Fig. 5

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: RHONE-POULENC AGROCHIMIE

(B) STREET: 14-20 Rue Pierre BAIZET

(C) TOWN: LYONS

(E) COUNTRY: France

(F) POST CODE: 69009

(ii) TITLE OF THE INVENTION: Gene coding for androctonine, vector containing it and disease-resistant transformed plants obtained

(iii) NUMBER OF SEQUENCES: 11

(vi) COMPUTER-READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 110 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..75

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AGG TCC GTG TGC AGG CAG ATC AAG ATC TGC AGG AGG AGG GGT GGT TGC  48
Arg Ser Val Cys Arg Gln Ile Lys Ile Cys Arg Arg Arg Gly Gly Cys
 1               5                  10                  15

TAC TAC AAG TGC ACT AAC AGG CCA TAC TGAGCTCGGC GAGGCGAACG        95
Tyr Tyr Lys Cys Thr Asn Arg Pro Tyr
            20                  25

TGTCGACGGA TCCGG                                               110
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 106 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 12..101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GCGTCGACGC C ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT 50
             Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu
               1               5                  10

CTT GTG TCT ACT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT  98
Leu Val Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg
    15                  20                  25

GCC GGCGA                                                      106
Ala
 30
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 211 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 12..176

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GCGTCGACGC C ATG GGT TTC GTG CTT TTC TCT CAG CTT CCA TCT TTC CTT   50
             Met Gly Phe Val Leu Phe Ser Gln Leu Pro Ser Phe Leu
               1               5                  10

CTT GTG TCT ACT CTT CTT CTT TTC CTT GTG ATC TCT CAC TCT TGC CGT   98
Leu Val Ser Thr Leu Leu Leu Phe Leu Val Ile Ser His Ser Cys Arg
    15                  20                  25

GCC AGG TCC GTG TGC AGG CAG ATC AAG ATC TGC AGG AGG AGG GGT GGT   146
Ala Arg Ser Val Cys Arg Gln Ile Lys Ile Cys Arg Arg Arg Gly Gly
 30                  35                  40                  45

TGC TAC TAC AAG TGC ACT AAC AGG CCA TAC TGAGCTCGGC GAGGCGAACG     196
Cys Tyr Tyr Lys Cys Thr Asn Arg Pro Tyr
                50                  55

TGTCGACGGA TCCGG                                                 211
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 75 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGTCGACGC GATGGGTTTC GTGCTTTTCT CTCAGCTTCC ATCTTTCCTT CTTGTGTCTA 60

CTCTTCTTCT TTTCC 75

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 72 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TCGCCGGCAC GGCAAGAGTA AGAGATCACA AGGAAAAGAA GAAGAGTAGA CACAAGAAGG 60

AAAGATGGAA GC 72

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 44 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 3"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 97 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 4"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CCGGATCCGT CGACACGTTC GCCTCGCCGA GCTCAGTATG GCCTGTTAGT GCACTTGTAG 60

TAGCAACCAC CCCTCCTCCT GCAGATCTTG ATCTGCC 97

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 85 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 5"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC 60

CTCTAGAGTC GACCTGCAGG CATGC 85

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 66 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic

oligonucleotide 6"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCCTGAACCA GGCTCGAGGG CGCGCCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC 60

TAGAGG 66

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 7"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCGGCCAGTC AGGCCACACT TAATTAAGTT TAAACGCGGC CCCGGCGCGC CTAGGTGTGT 60

GCTCGAGGGC CCAACCTCAG TACCTGGTTC AGG 93

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 93 base pairs

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "synthetic oligonucleotide 8"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGGCCTGAA CCAGGTACTG AGGTTGGGCC CTCGAGCACA CACCTAGGCG CGCCGGGGCC 60

GCGTTTAAAC TTAATTAAGT GTGGCCTGAC TGG 93

PCT/FR 98/01814

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C07K14/435 C12N15/12 A01H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | L. EHRET-SABATIER ET AL.,: "Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 47, 1996, pages 29537-29544, XP002060972 BETHESDA, MD, US cited in the application see the whole document -/-- | 1-8, 11-15, 18-21, 23-39 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 14 December 1998 | 21/12/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | MATEO ROSELL, A |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | EP 0 392 225 A (CIBA GEIGY AG)<br>17 October 1990<br><br>see abstract<br>see page 5, line 6 - page 6, line 31;<br>example 8<br>see page 23 | 1-8,<br>11-15,<br>18-21,<br>23-39 |
| A | WO 95 19443 A (CIBA GEIGY AG) 20 July 1995<br>cited in the application<br>see page 7, paragraph 3 - page 9,<br>paragraph 2<br>see sequence 17, page 64-65<br>see page 41, paragraph 44 | 12-17 |
| A | WO 95 11305 A (ZENECA LTD) 27 April 1995<br>see the whole document | 1-39 |
| A | EP 0 508 909 A (RHONE POULENC AGROCHIMIE)<br>14 October 1992<br>cited in the application<br>see the whole document | 1,19,<br>22-33 |
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A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6 C12N15/82 C07K14/435 C12N15/12 A01H1/00

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE

Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6 C12N C07K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

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[X] Voir la suite du cadre C pour la fin de la liste des documents

[X] Les documents de familles de brevets sont indiqués en annexe

* Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

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"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

| Date à laquelle la recherche internationale a été effectivement achevée | Date d'expédition du présent rapport de recherche internationale |
|---|---|
| 14 décembre 1998 | 21/12/1998 |

| Nom et adresse postale de l'administration chargée de la recherche internationale | Fonctionnaire autorisé |
|---|---|
| Office Européen des Brevets, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | MATEO ROSELL, A |

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